Computational pipeline for full brain histology to MRI registration

TUTORIAL GUIDE
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By using this pipeline, please cite:

1. Alegro, M. *et al*. Multimodal Whole Brain Registration: MRI and High Resolution Histology. http://www.cv-foundation.org/openaccess/content_cvpr_2016_workshops/w15/papers/Alegro_Multimodal_Whole_Brain_CVPR_2016_paper.pdf (2016).
2. Alho, E. J. L. *et al*. High thickness histological sections as alternative to study the three-dimensional microscopic human sub-cortical neuroanatomy. *Brain Struct. Funct.* (2017) doi:10.1007/s00429-017-1548-2.

Softwares: Matlab R2016b, ANTs (Advanced Normalization Tools), FSL, Freeview, Amira 5.5.0, Adobe Photoshop CS6, RectLabel

1. Pre-processing steps

1.1 Blockface background segmentation
1.2 Histology background segmentation
1.3 MRI pre-processing (field inhomogeneity correction, brain segmentation and resampling)

2. Processing steps

2.1 Histology to blockface registration (2D)
2.2 Alternative: Aligning slices
2.2 Histological 3D volume construction and intensity correction
2.3 Histology to MRI registration (3D)

3. Application and registration metrics

3.1 Structures segmentation in high resolution histology, mask creation and histology/MRI structure correlation
3.2 Three-dimensional structure reconstruction
3.3 Normalization to MNI stereotactic space
3.4 Registration metrics (Dice Similariy Coefficient –DSC and normalized weighted Spectral distance-nWSD)

Pipeline script content
Folders:
mri_pre-processing

dicm2nii
N4.m
Resample_image.m
MRI_ICBM152

registration

Apply_2DT.m
apply_3DT_general.m
build_histo_volume.m
center_volume.m
Reg2D.m
Reg3D.m
other auxiliary scripts

Segmentation_and_alignment

align_slices.m
align_slices2.m
Blockface_segmentation.m
color_align.m
histo_segmentation.m
refine_seg.m
Segmentation_for_1_img.m

Tools

crop_images.m
color_intensity.m
grayscale.m
histo_intensity_compensation.m
jpg2tif.m
jpg2tif2.m
plusone.m
rename_better.m
resize_masks.m
white2black.m
white2black_rgb.m
all_black.m
Preprocess_masks.m
BW.m
iso2mesh-master

Metrics

Dice_2D.m
Dice_3D.m
WESD_2D.m
HausdorffDist.m
auxiliary functions

This tutorial aims to guide how to apply a computational pipeline for full brain histology to MRI registration and how to apply the transforms generated in the registration procedure to masks segmented from high resolution histology. Normalization into MNI stereotactic space is also demonstrated. The great majority of algorithms is implemented in Matlab, therefore is expected from the user to have some familiarity with this environment. The variables used in each script are also documented in the scripts themselves.

Important: some Matlab scripts use comands to run programs outside Matlab and do not run properly if Matlab is opened from the desktop icon. Matlab should be opened through the Terminal window (type: open –a MATLAB_R2015 or the Matlab version you have on your computer)

1. Pre-processing steps

The registration procedure requires some pre-processing of the original images. Useless background information is removed from blockface and histological images (segmentation), and they are resized to have the same resolution and transformed to grayscale, in order to optimize 2D registration.

Before starting:

Please transform all .jpg images into .tif (to avoid lossy compression during downsampling and processing) with *jpg2tif2.m*. This script replaces the original .jpg images by .tif, so, be sure that this should be the format or copy the original images to a new folder before. The script *jpg2tif2.m* can deal with multiple format names, including words, spaces or simbols. On the other hand, the script *jpg2tif.m* only tranforms names with numbers on it, so it is preferable to use *jpg2tif2.m*. Using this script requires that you set your matlab directory to the folder where the .jpg images are.

Before going to the segmentation script, you can also crop the images to remove excessive background. This is not mandatory and you can crop them after if there is some extra information you want to remove. To acomplish this, run the script *crop_images.m* .

Organize the folders in a root folder containing 3 sets of images and name them as follows:

Blockface
Histology
MRI

This names will be important because they are part of the scripts.

1.1 Blockface background segmentation

To remove background of blockface images, run the script *Blockface_segmentation.m* . This script applies color based segmentation using k-means clustering and the variables here are:

root_dir: complete directory adress of the folder in which blockface images are located

sigma: standard deviation of gaussian distribution for gaussian blur filter. The higher this number is, more blur, which means more smooth segmented surface in the end of segmentation. Be careful, high numbers make results unreal. 5 is a good start for blockface segmentation. If the images are not smooth enough, higher values shoud be used, if they are too smooth, smaller.
clusters: number of clusters of colors the image shoud be divided. 2 or 3 are good starts.
resize: set 1 for image downsampling in 0.15 or [ ] to keep original size.

After running this function, a subfolder called *segmented* will be created inside the root_dir. Have a look on the images and see if the parameters you set could perform a good segmentation. If not, erase the segmentation folder and start over with new parameters. A good tip is to perform a trial in a few images copied to another folder. The final part of this script (line 77) transforms the cluster into a white mask using im2bw function. The threshold is set by default for blockface in 0.2, but you can vary this also for better results.

If a few images are not ideal, you can take them to another folder, set new parameters and run just the “bad”ones. If you got a good result, but some darker or lighter halo of the celloidin is still bothering, you can run the script *refine_seg.m*. This script uses the luminescence layer of the LAB color space to refine this darker or lighter edges. The parameters here are *root_dir* (folder where images are), *thresh (* threshold for segmentation, try 0.2 or 0.5, this depends on the image) and *sigma* (same parameter from the previous script (5 is a good start).

For the final blockface pre-processing, transform the segmented images to grayscale, using the *grayscale.m* function.

1.2 Histology background segmentation

To iniciate histology segmentation, also transform .jpg images into .tif using *jpg2tif2.m* . After doing that, run *histo_segmentation.m* . This script uses the same parameters used in blockface segmentation, but also has a new parameter called *resol* . This is the resolution paramenter, that should be the final resolution of the blockface images after all segmentation process. Just open a final blockface image and obtain the resolution, for instance if the dimensions are 843 x 562 the *resol* parameter should be [562 843] (inverse order). For the final histology pre-processing, transform the segmented images to grayscale, using the *grayscale.m* function.

Depending on the histological pictures, a white background inside the segmented area can remain. If that is the case, apply *white2black.m* to the grayscaled images or *white2black_rgb.m* if they are rgb images.

If either histological or blockface images from the beginning or end of the stack are with bad segmentation, simply remove them, but the number of images in Blockface and Histology folders must remain the same .

1.3 MRI pre-processing (field inhomogeneity correction, brain segmentation and resampling)

The first step in MRI pre-processing is transforming DICOM files into Nifti format. You can acomplish this by typing *dicm2nii* in your Matlab command line. A GUI will open and you shoud choose the DICOM file folder and the directory where the nifti files should be stored.
After that, several folders with the different MRI sequences are to be found.
The next step is field inhomogeneity correction. The N4 algorithm is used, use the *N4.m* Matlab script to run the ANTs command. The settings can be changed for a better result, details about the variables are described within the script.

After doing this, skull stripping shoul be performed. Type “fsl” in the Terminal command line and use BET brain extration for that . We recommend setting threshold to 0.75 and use the option Robust brain centre estimation. Higher threshold values will ‘eat’ the brain, lower can leave undesired parts. The result will be in the format .nii.gz. To unzip it, you can open the volume in Freeview (to view the result) and save as .nii format. Another way to transform to .nii is type on the Terminal command line:

```
cd /directory/where/the/mri/is
mri_convert name.mgz name.nii
```

Last step is upsampling the MRI to a higher resolution (0.33x0.33x0.33). To do this, use the *Resample_Image.m* script.

2. Processing steps

2.1 Histology to blockface registration

In order to account for 2D deformations and alignment of the histology to the blockface images, use the script *Reg2D.m*. Input is a *root_dir*. Inside this folder, the images to be registered should be placed inside folders called:

Blockface/segmented/grayscale/
Histology/segmented/grayscale/

By default, inicial alignment is performed with mri_robust_register. convert to .mgz and ANTs non-linear registrations are options, but should be set if wanted (do MGZ=1 instead of 0 -line 49- and do ANTS = 1 instead of 0, line 51). Note that for the ANTs non linear registration to work properly, very good blockface segmentation should be achieved, otherwise the histological slices will be streched in an unrealistic way.

When applying *Reg2D.m*, the following folders are going to be created:

Histology/reg1 (where .lta files, logs and, nii.gz archives are stored)
Histology/regblock (.tif transformed images)

Two alternative methods can be used when histology to blockface registration show suboptimal results. Both of these methods consist in aligning the histological slices intrisecally, instead of registering the images. This alignment can be achieved either in the *align_slices.m* script, that uses mri_robust_register to align a pair of slices, or Amira. In the case of using Amira, connect the module AlignSlices to the desired .tif volume, and align the slices with the option Save Transforms clicked. Doing this, it is possible to conect other volumes (for instance masks) and apply the same transforms to these masks, by conecting the masks (should be equal resolution and number of slices) to the previously aligned volume. Save the whole reconstruction in Save Network as pack and go". You can export the aligned .tif slices by clicking on the volume, Save as, 2D tif. Watch out the numbering (starts from 000.tif or ###.tif and not 001.tif , as our slices do). To overcome this, export in Amira as ###.tif, and then apply the *plusone.m* script, that will add 001 in each slice.
If you use *align_slices.m* the script will choose the middle of the stack to start the aligning process. If some error occur and you want to align just a few slices, starting with the last one, please use *align_slices2.m* .

It is possible to apply the .lta transforms obtained in *align_slices.m* to RGB images using *color_align.m*.

2.2 Histological 3D volume construction and intensity correction

First of all, the histological voxel size must be calculated. This is done by hand. Open a histological image with a fotographed ruler in a program where pixels can be counted (Photoshop, for example). The dimensions should be the final, after all the resampling. The x and y dimensions should be the same (as they are isotropic pictures). Count how many pixels in the x and y direction there are. For example, if in 10 mm there are 45 pixels, 1 pixel is equivalent to 0.220 mm (10/45). As a rule, x and y pixel sizes are given by 1 divided by how many pixels are found in 1 mm of the picture, but it is better to measure in 10mm and divide 10/pixel number. So, x and y dimensions are 0.220 and the z dimension is the thickness of the slice in mm. In the case of 430 micrometers, 0.430. For this case, the voxel dimensions are [0.220 0.220 0.430].

Histological slices may have gray value/color intensity differences among each other, so when a 3D volume is reconstructions, stripes appear. To get rid of these stripes, intensity correction should be proceded. Please run the script *histo_intensity_compensation.m* in the .tif histological aligned grayscaled images.

If the images are RGB, choose *color_intensity.m*. You will have to choose one image as reference to all the others as a parameter to correct the intensity.

After the correction, use *build_histo_volume.m*. It will generate a .mgz and .nii volumes (histo_volume.nii/.mgz) ready to register to the pre-processed MRI. The inputs are the image folder and voxel size calculated with the method above.

2.3 Histology to MRI registration (3D)

2.3.1 Rough center alignment

Before starting registration, we must manually center the histological volume with the MRI volume (they are in very different spaces). Open the MRI upsampled skull striped volume in Freeview and the histo_volume.nii. They will appear in different places, you must align them manually with the tool Transform Volume (Tools). Apply translations and rotations until the volumes are in the same space, and save the registration file (Save reg). This will save a .lta file, wich can be opened in a text editor. Save this transform as M1.lta. Most probably you will need more than 1 transformation to achieve a good result (since the histological volume gets out of the field of view, and you will have to stop. If this is the case, keep doing it paciently and storing M2, M3....until it is done. After each transform, you have to apply it to the volume. Go to Matlab and open the script *center_volume.m* and follow the instructions. Save the final transform script with another name for future use.

2.3.2 ANTs registration

After the volumes are roughly aligned, run the *Reg3D.m* script .This script will register the histological volume to the MRI volume and store the transformations for future use in a folder called 3Dtransforms.

Open the volumes in Freeview to check the final 3D registration.

3. Application and registration metrics

3.1 Structures segmentation in high resolution histology, mask creation and histology/MRI structure correlation

After the registration process, the final histological volume registered to the MRI has an intermediate resolution of 0.33x0.33x0.33mm3. This resolution is high in terms of MRI, but low compared to the original histological pictures. Segmentation of big structures like the subthalamic nucleus is possible on this resolution, but small structures or structures that require high contrast or dark field microscopy should be segmented in the original slices and transfered to the MRI through application of the transforms generated in the process.

3.1.1 Segmentation of structures in high resolution histology in Photoshop CS6

Open the original stained histological slice on Photoshop. If there is a corresponding darkfield illumination picture, open it also, copy and paste as a layer and use the menu Edit-Transform to register the dark field into the image. You can perform this action for all the slices you want before start segmenting.

For the segmentation, create layers with the names of the structures you are segmenting (Layer-New-Layer), you can automate this action using control keys (in the window actions, create a new set, then new action and associate a function key to this action. Click record, and you will record all the actions while the record button is pressed. To terminate recording, press stop).

Select the layer with the structure you want to segment, choose the pencil tool, pick up the black color and set the pencil siz to 5 pixels. You can set bigger or smaller sizes if you desire. Draw the structure, the most external border of the structure should coincide with the most external border of the pencil (that means, the pencil's thickness will count as part of the structure and not outside).You can use the eraser to correct mistakes. Remember to close the structures. Move from layer to layer until you are done. Save as .psd (or tiff) to have all layers embedded and then save each layer separately, disabling all other layers (clicking on the eye icon), leaving just the mask you want to save. Save as .tif, with the same number as the original, with 3 digits (001.tif), but in a proper folder.

This procedure will save a black and white mask, but you will have to invert the colors. Open the saved mask, click on Image-adjustments-invert and save the inverted mask. You can Batch this procedure for a whole folder, by recording this action, then go to File-automate-Batch, choose the folders and click ok.

3.1.2 Mask creation

At this moment, you will have folders named with the desired structures, but differing in numbers of slices. Let's say, a folder named *caudate* and another named *GPi*. You must create a folder with black .tif images in the same resolution of the original slices. To do this, create a new folder called black_mask and copy the original histological images to it. If this images are not in .tif , please run the *jpg2tif2.m* and then apply the *all_black.m* script. You will get a folder full of numbered black masks. You will use this masks to complement the other folders. For instance, if the folder caudate has images from 145.tif to 198.tif, then copy black slices from 001.tif to 144.tif and from 199.tif until the last image, in a way that all mask folders have the same number of slices.

To prepare the masks to be registered, run *Preprocess_masks.m* . Check if the final images have the same size of the registered histology (number of starting slice, end slice and number of slices) and if all the masks are filled with white. If yes, they are ready for the next step.

Alternatively, masks can be created within other softwares, as RectLabel.

3.1.3 Histology to MRI structure correlation

When the masks are ready, they will be on a folder called preprocessed_masks_name (name will be the name of the mask to be transformed).

3.1.3.1 Applying 2D transforms

Now there are 3 possibilities:

On the first, you performed 2D ANTs histology registration to blockface in order to obtain an aligned histological set. If this is the case, you will apply the *Apply_2DT.m,* using the .lta files originated in the registration process.

Second possibility, you aligned the histological set with *align_slices.m* and in this case, you will proceed exacty the same way using *Apply_2DT.m* (but the .lta files will be from the alignment and not histo to blockface registration.

On the 3rd case, you aligned the slices with Amira. In this case, you have to open the Amira .hx file in which you stored the alignment, open data, choose the masks, attacht AlignSlices, click on the white square and connect the original aligned slices as the reference, then resample. Check the result, save as 2D tif, in the format ###.tif and then apply the *plusone.m* script to have a 2D aligned set ready for 3D transformation.

3.1.3.2 Applying 3D transforms

To transform the masks into the 3D registered volume, run *apply_3DT_general.m* and follow the arguments in the script. If you have more then 1 mask to apply, have a look in the Scripts_case01 folder, in the *Apply_multiple3DT.m* and modify it for your personal use.

3.2 Three-dimensional structure reconstruction

You can visualize the results on Freeview or in Amira, generating 3D structures.

Open data, connect a label field, click on the white mask, with all slices option on, segment them all, then use surfaceGen and SurfaceView to generate 3D images and visualize them on 3D space. See Amira help for detailed information about this process.

3.3 Normalization to MNI stereotactic space

It is sometimes usefull to normalize your data to a common standard space. We use here the MNI stereotactic space, namely the ICBM152. In the mri registration folder, you will find the MNI ICBM152 images. To register with the T1, use *mni_icbm152_t1_tal_nlin_asym_09c.nii*. First use the *Resample_Image.m* to upsample the MNI MRI to 0.33x0.33x0.33 resolution.

After that, use the *Reg3D.m* to register the Case MRI into the MNI MRI. Use the *apply_3DT_general.m* to apply the normalization to the histological volume and to the masks.

You can visualize the results in Amira and Freeview.

3.4 Registration metrics (Dice Similariy Coefficient –DSC and Weighted Spectral distance-WESD)

The Dice Similarity Coefficient (DSC) is used to quantify the amount of overlap between MRI and registered histology (DSC range is in the [0, 1] interval where 0 means no overlap and 1 means maximum overlap). We additionally employed the weighted spectral Distance (WESD) to quantify shape dissimilarities between the MRI and histology segmentations (WESD range is in the [0, 1] interval, where 0 means a perfect match between shapes while 1 means complete disagreement).

To compute these values, create the binary masks you want to compare (you can use *BW.m* to transform grayscale or rgb images into binary images) and use *Dice_2D.m* to compute Dice coefficient of 2D slices. The mean, standard deviation, an excel table and a graphic are outputs of all these functions. Means of 2D Dice and WESD 2D correspond approximately to Dice 3D and WESD 3D. To compute Dice 3D you can use *Dice_3D.m* and to compute WESD use *WESD_2D.m*.